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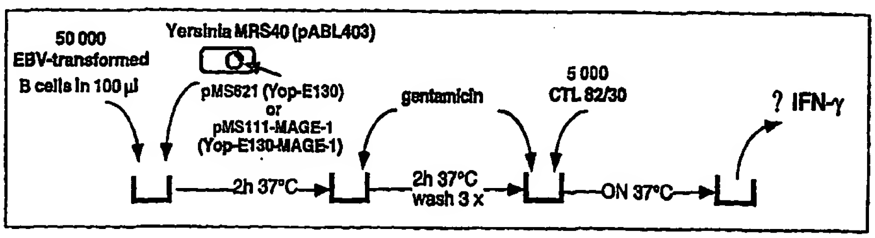

(57) Abstract

The present invention relates to recombinant *Yersinia* and the use thereof for delivery of proteins into eukaryotic cells, including related compositions and methods of treatment and related assays.

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DELIVERY OF PROTEINS INTO EUKARYOTIC CELLS WITH RECOMBINANT *YERSINIA*

The present invention relates to recombinant *Yersinia* and the use thereof for delivery of proteins into eukaryotic cells.

Bacteria of the genus *Yersinia* cause diseases in humans and rodents ranging from enteritis and lymphadenitis to plague. The genus *Yersinia* encompasses three species: *Yersinia enterocolitica*, which is the most prevalent *Yersinia* species in humans and causes a broad range of gastro-intestinal syndromes; *Yersinia pseudotuberculosis*, which causes adenitis and septicaemia; and *Yersinia pestis*, which is the causative agent of plague.

In spite of the differences in the infection routes, these three species of *Yersinia* share a common capacity to resist the non-specific immune response of the human or rodent host and to proliferate in the host lymphatic tissues. Anatomo-pathological examinations revealed that *Yersinia* are not detected inside the inflammatory or parenchymal cells of the infected animals (Simonet et al. (1990) *Infect. Immun. 58*: 841-845). Consistent with these in vivo observations, *Yersinia* are resistant to phagocytosis in vitro by macrophages and polymorphonuclear leukocytes. See review by Cornelis et al. (1997) *Mol. Microbiol. 23(5)*: 861-867. *Yersinia enterocolitica* also has the capacity to enter certain cultured epithelial cells, a process generally referred to as

invasion (Miller et al. (1988) *Infect. Immun.* 56: 1242-1248).

Genetic studies revealed that the virulence of *Yersinia* is determined by a 70 kb plasmid (pYV), which encodes and governs the production of a set of proteins called Yops (for *Yersinia* outer proteins). These Yops form an integrated anti-host system that allows the extracellular adhesion of *Yersinia* to the surface of host cells and the subsequent injection of a set of toxic effector proteins into the host cell's cytosol. Recent studies further revealed that such an anti-host system, also called "*Yersinia* virulon", is composed of the following four elements: (i) a contact or type III secretion system called Ysc, which is devoted to the secretion of Yop proteins out of the bacterial cells; (ii) a set of "translocators" for translocating the effector proteins into the eukaryotic host cells, which consist of YopB, YopD and possible other proteins such as LcrV; (iii) a control element and recognition system (YopN and LcrG); and (iv) a set of "effector proteins" including YopE, YopH, YopO/YpkA, YopM and YopP/YopJ, which are injected (or translocated) into the eukaryotic host cells and disrupt the functions of such host cells. Transcription of these genes is controlled both by temperature and by contact with a eukaryotic cell. See review by Cornelis et al. (1997).

The effector proteins disrupt the function of host cells in a number of ways. The 23 kd YopE is a cytotoxin that disrupts the actin-microfilament structure of cultured Hela cells (Rosqvist et al.

(1990) *Mol. Microbiol. 4*: 657-667; Rosqvist et al. (1991) *Infect. Immun. 59*: 4562-4569). The 51 kd YopH is a protein tyrosine phosphatase (PTPase) related to eukaryotic PTPases, which acts on tyrosine-phosphorylated proteins of infected macrophages (Hartland et al. (1994) *Infect. Immun. 62*: 4445-4453). Presumably as a result of this action, YopH inhibits bacterial uptake and oxidative burst by cultured macrophages (Rosquvist et al. (1988) *Infect. Immun. 56*: 2139-2143; Bliska et al. (1995) *Infect. Immun. 63*: 681-685). YopO (or YpkA) is an 81 kd serine/threonine kinase, which is targeted to the inner surface of the plasma membrane of the eukaryotic cell and might function to interfere with the signal transduction pathway of the eukaryotic cell (Hakansson et al. (1996) *Mol. Microbiol. 20*: 593-603). YopM is an acidic 41 kd protein having 12 leucine-rich repeats, which suggests that YopM might bind thrombin and interfere with platelet-mediated events of the inflammatory response (Leung et al. (1989) *J. Bacterial. 171*: 4623-4632). YopP is involved in the induction of apoptosis in macrophages (Mills et al. (1997) Proc. Acad. Natl. Sci. USA 94: 12638-12643).

The molecular structures of these effector proteins have been investigated to determine the elements in each effector protein that are required for their secretion and translocation. For this purpose, hybrid proteins have been engineered by fusing truncated Yop effector proteins of different length with certain reporter enzymes such as the calmodulin-activated adenylate cyclase domain (or Cya)

of the *Bordetella pertussis* cyclolysin. Successful secretion and/or translocation events could be detected by assays based on the enzymatic activity of the reporter protein. Sory et al. disclose, by applying this approach, that YopE and YopH of *Y. enterocolitica* are modular proteins composed of three domains, i.e.,an N-terminal domain required for secretion, a translocation domain required for translocation into cells, and a C-terminal catalytic domain responsible for the toxic effector activity. Sory et al. (1995) *Proc. Natl. Acad. Sci. USA 92*: 11998-12002. The same domain organization has been demonstrated for YopM of *Y. enterocolitca* (Boland et al. (1996) *EMBO J. 15*: 5191-5201).

The present invention provides recombinant *Yersinia* for safe delivery of proteins into eukaryotic cells. Such *Yersinia* are deficient in the production of functional effector proteins, but are endowed with a functional secretion and translocation system. The present invention further provides expression vectors for use in combination with such mutant *Yersinia* for safe and efficient delivery of desired proteins into eukaryotic cells. This approach is useful not only for studying the function of a given protein, but also for designing therapeutic approaches. For example, a protein of a pathogenic origin, e.g., a tumor associated protein, a parasite antigen, or a viral antigen, can be delivered using the recombinant *Yersinia* of the present invention into antigen presenting cells for inducing an immune response specific for such a protein.

Most progressively growing neoplastic cells express potentially immunogenic tumor-associated antigens (TAAs), also called tumor rejection antigens (TRAs). TRAs, like other antigenic epitopes, are presented at the surface of tumor cells by MHC molecules and have been shown to induce a CTL response *in vivo* and *in vitro*. See, for example, van der Bruggen et al. (1991) *Science 254*: 1643-1647. However, such TRA-expressing tumor cells do not provoke reliable anti-tumor immune responses *in vivo* that are capable of controlling the growth of malignant cells. Boon et al. (1992) *Cancer Surveys 13*: 23-37; T. Boon (1993) *Int. J. Cancer 54*: 177-180; T. Boon (1992) *Advances Cancer Res. 58*: 177-209.

A number of genes have been identified that encode tumor rejection antigen precursors (or TRAPs), which are processed into TRAs in tumor cells. Such TRAP-encoding genes include members of the MAGE family, the BAGE family, the DAGE/Prame family, the GAGE family, the RAGE family, the SMAGE family, NAG, Tyrosinase, Melan-A/MART-1, gp100, MUC-1, TAG-72, CA125, mutated proto-oncogenes such as p21ras, mutated tumor suppressor genes such as p53, tumor associated viral antigens such as HPV16 E7. See, e.g., review by Van den Eynde and van der Bruggen (1997) in *Curr. Opin. Immunol.* 9:684-693, Sahin et al. (1997) in *Curr. Opin. Immunol.* 9:709-716, and Shawler et al. (1997) *Advances in Pharmacology 40*: 309-337 Academic Press, Inc.: San Diego, California. The identification of these genes has allowed recombinant production of TRAs

or TRAPs which may be subsequently used as vaccines to treat various cancerous conditions.

The present invention contemplates the use of recombinant *Yersinia* for delivery of desired proteins into eukaryotic cells. Particularly, the recombinant *Yersinia* of the present invention are useful for delivery of proteins or derivatives thereof to antigen presenting cells. In accordance with the present invention, antigen presenting cells upon receiving the delivery, present antigenic epitopes which can be recognized by T cells. Thus, the recombinant *Yersinia* of the present invention can be employed in a number of immune diagnostic or therapeutic approaches.

The present invention is further elaborated upon the following disclosure.

The present invention relates to recombinant *Yersinia* and the use thereof for delivery of proteins into eukaryotic cells.

One embodiment of the present invention provides mutant *Yersinia* strains deficient in producing functional effector proteins. A preferred mutant *Yersinia* strain of the present invention is a quintuple-mutant strain designated as *yopEHMOP*.

Another embodiment of the present invention provides expression vectors for delivery of heterologous proteins to eukaryotic cells. In accordance with the present invention, such an expression vector is characterized by (in the 5' to 3' direction) a promoter, a first nucleic acid sequence encoding a delivery signal, a second nucleic acid

sequence fused thereto coding for a heterologous protein to be delivered.

According to this embodiment of the present invention, the promoter is preferably one from a *Yersinia* virulon gene; more preferably, an effector-encoding gene; even more preferably, a *YopE* gene. The delivery signal is a polypeptide sequence from an *Yersinia* effector, including YopE, YopH, YopO/YpkA, YopM, and YopP/YopJ. Such delivery signal can be recognized by the secretion and translocation system of *Yersinia*. The heterologous protein of the present invention includes naturally occurring proteins or parts thereof such as tumor-associated proteins or known antigens of pathogens. The heterologous protein of the present invention also includes artificially designed proteins such as in-frame fusion of proteins or parts of proteins.

Yet another embodiment of the present invention provides recombinant *Yersinia*, i.e., yersinia of the above-described mutant strains further transformed with the expression vector of the present invention. Such recombinant *Yersinia* is preferred for delivery of heterologous proteins into eukaryotic cells. A preferred eukaryotic cell is an antigen presenting cell capable of presenting immunogenic epitopes derived from the heterologous proteins being delivered.

In a further aspect of the present invention, such recombinant *Yersinia* are contemplated in immunogenic compositions and methods for inducing an immune response, either a cellualr immune response

or a humoral immune response, or a combination of both.

Further to this aspect of the invention, the recombinant *Yersinia* of the present invention can be employed in an *in vitro* regime for assessing the efficacy of a vaccination regimen. The recombinant *Yersinia* of the present invention can also be employed in an *ex vivo* regime to generate specific CTLs and to use such CTLs for treating various pathological conditions such as tumors or infections by pathogens. The recombinant *Yersinia* of the present invention can also be employed in an *in vivo* regime, i.e., as a recombinant vaccine, to treat subjects suffering pathological conditions such as tumors or infections by pathogens.

Figure 1 illustrates the plasmid map of the expression vector pMS111-MAGE-1 ($YopE_{130}$-MAGE1).

Figure 2 (A) depicts the procedure for stimulating CTL 82/30 with EBV-transformed human B cells (HLA-A1) mixed wth recombinant *Yersinia*; (B) depicts the quantitation of IFN-γ released by activated CTLs.

Figure 3 depicts the sequence of the *Yersinia enterocolitica YopM* gene.

Figure 4 depicts the sequence of the *Yersinia enterocolitica YopE* gene.

Figure 5 depicts the sequence of the *Yersinia enterocolitica YopH* gene.

Figure 6 depicts the sequence of the *Yersinia enterocolitica YopP* gene.

Figure 7 depicts the sequence of the *Yersinia enterocolitica YopP* gene.

The present invention relates to recombinant *Yersinia* and the use thereof for delivery of proteins into eukaryotic cells.

In particular, the present invention provides mutant *Yersinia* strains that are deficient in producing functional effector proteins. The present invention further provides an expression vector, which, when transformed into a *Yersinia* of the above-described mutant strains, permits delivery of a desired protein, e.g., a MAGE-1 protein, into a eukaryotic cell, for example, a EBV-transformed human B cell. The present invention provides that, upon receiving the delivered protein, the antigen presenting cell processes the delivered protein and presents antigenic epitopes in the context of an MHC molecule leading to an immune response specific for the protein being delivered. For example, human B cells after taking up the MAGE-1 protein from a recombinant *Yersinia* of the present invention are recognized by a CTL clone specific for a MAGE-1 epitope. Thus the present invention provides an effective recombinant *Yersinia* system with diminished toxicity for delivery of desired proteins into eukaryotic cells.

The term "*Yersinia*" as used herein means all species of Yersinia, including *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Yersinia pestis*.

For the purpose of the present invention, the term "recombinant *Yersinia*" used herein refers to *Yersinia* genetically transformed with the expression vectors of the present invention.

The term "delivery" used herein refers to the transportation of a protein from a *Yersinia* to a eukaryotic cell, including the steps of expressing the protein in the *Yersinia*, secreting the expressed protein(s) from such *Yersinia* and translocating the secreted protein(s) by such *Yersinia* into the cytosol of the eukaryotic cell. Accordingly, a "delivery signal" refers to a polypeptide sequence which can be recognized by the secretion and translocation system of *Yersinia* and directs the delivery of a protein from *Yersinia* to eukaryotic cells.

As used herein, the "secretion" of a protein refers to the transportation of such protein outward across the cell membrane of a *Yersinia*. The "translocation" of a protein refers to the transportation of such protein across the plasma membrane of a eukaryotic cell into the cytosol of such eukaryotic cell.

"Eukaryotic cells" as used herein, the surface of which *Yersinia* adhere to, are also referred to as "target cells" or "target eukaryotic cells".

One embodiment of the present invention provides mutant *Yersinia* strains which are deficient in producing functional effector proteins.

The effector proteins of *Yersinia*, i.e., the *Yersinia* virulon proteins which are normally translocated into the cytosol of the target eukaryotic

cells, are toxic to the target cell. Thus, a "functional effector protein" refers to an effector protein having a defined catalytic activity and which is capable of eliciting specific toxicity toward the target cells.

Accordingly, the mutant *Yersinia* of the present invention are used for delivery of proteins to eukaryotic cells, with diminished toxicity, i.e., toxicity which does not completely disable or kill the target cell. For the purpose of delivering proteins, the secretion and translocation system of the instant mutant *Yersinia* need to be intact.

Five effector genes have been cloned from *Y. enterocolitica* which are *YopE*, *YopH*, *YopO*, *YopM*, and *YopP* (Figures 3-7). The equivalent effector genes have been cloned from *Y. pseudotuberculosis* and are named as *YopE*, *YopH*, *YpkA*, *YopM*, and *YopJ*, respectively. Some effector genes have also been cloned from *Y. pestis*. The nucleic acid sequences of these *Yop* genes are available to those skilled in the art, e.g., in the Genebank Database

For the purpose of the present invention, the effector-encoding genes are denoted by italicized letters to be distinguished from the effector proteins. Mutant effector genes are denoted by letters of lower case. For example, YopE refers to the effector protein encoded by the *YopE* gene. *YopE* represents the wild type gene, while *yopE* represents a gene having a mutation.

According to the present invention, a mutant *Yersinia* strain can be generated by introducing at

least one mutation into at least one effector-encoding gene. Preferably, such effector-encoding genes include *YopE*, *YopH*, *YopO/YpkA*, *YopM*, and *YopP/YopJ*. The skilled artisan may employ any number of standard techniques to generate mutations in these *Yop* genes. Sambrook et al. describe in general such techniques. See Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual, Second Edition* Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York.

The term "mutation" is used herein as a general term and includes changes of both single base pair and multiple base pairs. Such mutations may include substitutions, frame-shift mutations, deletions and truncations.

In accordance with the present invention, the mutation can be generated in the promoter region of an effector-encoding gene so that the expression of such effector gene is abolished.

The mutation can also be generated in the coding region of an effector-encoding gene such that the catalytic activity of the encoded effector protein is abolished. The "catalytic activity" of an effector protein refers to the anti-target cell function of an effector protein, i.e., toxicity. Such activity is governed by the catalytic motifs in the catalytic domain of an effector protein. The approaches for identifying the catalytic domain and/or the catalytic motifs of an effector protein are well within the ken of those skilled in the art. See, for example, Sory et al. (1995), Boland et al. (1996) and Cornelis et al. (1997).

Accordingly, one preferred mutation of the present invention is a deletion of the entire catalytic domain. Another preferred mutation is a frameshift mutation in an effector-encoding gene such that the catalytic domain is not present in the protein product expressed from such "frameshifted" gene. A most preferred mutation is a mutation with the deletion of the entire coding region.

Other mutations are also contemplated by the present invention, such as small deletions or base pair substitutions, which are generated in the catalytic motifs of an effector protein leading to destruction of the catalytic activity of a given effector protein.

The mutations that are generated in the *Yop* genes may be introduced into *Yersinia* by a number of methods. One such method involves cloning a mutated *Yop* gene (i.e., a *yop* gene), into a "suicide" vector which is capable of introducing the mutated *yop* sequence into *Yersinia* via allelic exchange. Such "suicide" vectors are described by Kaniga et al. (1991) *Gene 109*: 137-141 and by Sarker et al. (1997) *Mol. Microbiol 23*: 409-411. In this manner, mutations generated in multiple *Yop* genes may be introduced successively into *Yersinia*, giving rise to polymutant recombinant *Yersinia*. The order in which these mutated *yop* sequences are introduced is not important.

A preferred mutant *Yersinia* strain of the present invention is a quintuple-mutant *Yersinia* strain in which all the effector-encoding genes are

mutated such that the resulting *Yersinia* no longer produce any functional effector proteins. Such quintuple-mutant *Yersinia* strain is designated as *yopEHOMP* for *Y. enterocolitica* or *yopEHAM* for *Y. pseudotuberculosis*. One example of such *yopEHOMP* strain is *Y. enterocolitica* MRS40(pABL403).

Under some circumstances, it may be desired to mutate only some but not all of the effector *Yop* genes. For example, when a delivery is intended to target a macrophage, *YopH* is preferably not mutated since YopH is understood to inhibit the phagocytosis by macrophages. Rosqvist et al. (1988) and Rosqvist et al. (1989). Accordingly, the present invention further contemplates polymutant *Yersinia* other than quintuple-mutant *Yersinia*, e.g., double-mutant, triple-mutant, and quadruple-mutant *Yersinia*.

Alternatively, the quintuple-mutant strain *yopEHOMP* may still be used for delivery to macrophages, in which case a wild type *YopH* gene can be introduced into the quintuple-mutant *Y. yopEHOMP* strain by various known transformation procedures which are further described hereinafter. In this manner, a polymutant *Yersinia* strain can be generated in which a desired set of *Yop* genes are mutated such that only the protein of interest is delivered into the target cells.

A further aspect of the present invention is directed to an expression vector for use in combination with the instant mutant *Yersinia* strains to deliver a desired protein into eukaryotic cells. In accordance with the present invention, such a

vector is characterized by (in the 5' to 3' direction) a promoter, a first nucleic acid sequence encoding a delivery signal, a second nucleic acid sequence fused thereto coding for a heterologous protein to be delivered.

In accordance with present invention, the promoter of the expression vector is preferably from a *Yersinia* virulon gene. A "*Yersinia* virulon gene" refers to genes on the *Yersinia* pYV plasmid, the expression of which is controlled both by temperature and by contact with a target cell. See review by Cornelis et al. (1997). Such genes include genes coding for elements of the secretion machinary (the *Ysc* genes), genes coding for translocators (*YopB*, *YopD*, and *LcrV*), genes coding for the control elements (*YopN* and *LcrG*), and genes coding for effectors (*YopE*, *YopH*, *YopO/YpkA*, *YopM* and *YopP/YopJ*).

In a preferred embodiment of the present invention, the promoter is from an effector-encoding gene selected from any one of *YopE*, *YopH*, *YopO/YpkA*, *YopM* and *YopP/YopJ*. More preferrably, the promoter is from *YopE*.

Further in accordance with the present invention, a first DNA sequence coding for a delivery signal is operably linked to the promoter.

"A delivery signal", as described hereinabove, refers to a polypeptide which can be recognized by the secretion and translocation system of *Yersinia* and therefore directs the secretion and translocation of a protein into a eukaryotic cell.

According to the present invention, such a polypeptide is from an effector protein. The effector proteins include YopE, YopH, YopO/YpkA, YopM, and YopP/YopJ. Preferably, the effector protein is YopE. More preferably, the effector protein is YopE of *Yersinia enterocolitica*.

One skilled in the art is familiar with the methods for identifying the polypeptide sequences of an effector protein that are capable of delivering a protein. For example, one such method is described by Sory et al. (1994). Briefly, polypeptide sequences from various portions of the Yop proteins can be fused in-frame to a reporter enzyme such as the calmodulin-activated adenylate cyclase domain (or Cya) of the *Bordetella pertussis* cyclolysin. Delivery of a Yop-Cya hybrid protein into the cytosol of eukaryotic cells is indicated by the appearance of cyclase activity in the infected eukaryotic cells that leads to the accumulation of cAMP. Examples of such delivery signal polypeptides include from *Y. enterocolitica*: $YopE_{130}$ (the N-terminal 130 amino acids of YopE), $YopE_{50}$, $YopM_{100}$ and $YopH_{71}$.

By employing such an approach, one skilled in the art can determine, if desired, the minimal sequence requirement, i.e., a contiguous amino acid sequence of the shortest length, that is capable of delivering a protein. See, e.g., Sory et al (1994). Accordingly, preferred delivery signals of the present invention consists of at least the minimal sequence of amino acids of a Yop effector protein that is capale of delivering a protein.

Further in accordance with the present invention, a second DNA sequence encoding a heterologous protein is fused in frame to the first DNA sequence in the instant vector for delivery into eukaryotic cells.

The term "heterologous protein" used herein refers to a protein other than a *Yersinia* Yop protein. "Yop proteins" refer to *Yersinia* virulon proteins that are secreted, including the translocators and the effectors.

According to the present invention, "a heterologous protein" includes naturally occuring proteins or parts thereof. The term "part of a protein" includes a peptide or polypeptide fragment of a protein that is of sufficient length to be antigenic. Preferably, such a fragment consists of at least 8 or 9 contiguous amino acids of a protein. "A heterologous protein" as used in the present invention also includes artificially engineered proteins or parts thereof, such as fusion of two or more naturally occurring proteins or parts thereof, polyepitopes (in-frame fusion of two or more peptide epitopes) as exemplified by Thompson et al. (1995) in *Proc. Natl. Acad. Sci. USA* 92: 5845-5849.

The protein expressed from the fused first and second DNA sequences is also termed as a "fusion protein" or a "hybrid protein", i.e., a hybrid of *Yersinia* delivery signal and a heterologous protein.

There is no particular limitation in the heterologous protein that can be delivered. The present invention particularly contemplates proteins,

such as, e.g., known tumor associated proteins, known antigens of pathogens, and cytokines.

A "tumor associated protein" refers to a protein that is specifically expressed in tumors or expressed at an abnormal level in tumors relative to normal tissues. Such tumor associated proteins include, but are not limited to, members of the MAGE family, the BAGE family (such as BAGE-1), the DAGE/Prame family (such as DAGE-1), the GAGE family, the RAGE family (such as RAGE-1), the SMAGE family, NAG, Tyrosinase, Melan-A/ MART-1, gp100, MUC-1, TAG-72, CA125, mutated proto-oncogenes such as p21ras, mutated tumor suppressor genes such as p53, tumor associated viral antigens (e.g., HPV16 E7), HOM-MEL-40, HOM-MEL-55, NY-COL-2, HOM-HD-397, HOM-RCC-1.14, HOM-HD-21, HOM-NSCLC-11, HOM-MEL-2.4, and HOM-TES-11. Members of the MAGE family include, but are not limited to, MAGE-1, MAGE-2, MAGE-11. Members of the GAGE family include, but are not limited to, GAGE-1, GAGE-6. See, e.g., review by Van den Eynde and van der Bruggen (1997) in *Curr. Opin. Immunol.* 9:684-693, Sahin et al. (1997) in *Curr. Opin. Immunol.* 9:709-716, and Shawler et al. (1997). These proteins have been shown to associate with certain tumors such as melanoma, lung cancer, prostate cancer, breast cancer, renal cancer and others.

A number of known antigens derived from pathogens can also be employed according to the present invention. Pathogens contemplated by the present invention include viruses, bacteria, parasites and fungi. Specific examples of antigens

characteristic of a pathogen include the influenza virus nucleoprotein (residues 218-226, as set forth in F. et al. (1997) *J. Virol. 71*: 2715-2721) antigens from Sendai virus and lymphocytic choriomeningitis virus (see, An et al. (1997) *J. Virol. 71*: 2292-2302), the B1 protein of hepatitis C virus (Bruna-Romero et al. (1997) *Hepatology 25*: 470-477), the virus envelope glycoprotein gp 160 of HIV (Achour et al. (1996) *J. Virol. 70*: 6741-6750), amino acids 252-260 or the circumsporozite protein of *Plasmodium berghei* (Allsopp et al. (1996) *Eur. J. Immunol. 26*: 1951-1958), the influenza A virus nucleoprotein (residues 366-374, Nomura et al. (1996) *J. Immunol. Methods 193*: 4149), the listeriolysin O protein of *Listeria monocytogenes* (residues 91-99, An et al. (1996) *Infect. Immun. 64*: 1685-1693), the E6 protein (residues 131-140, Gao et al. (1995) *J. Immunol. 155*: 5519-5526) and E7 protein (residues 21-28 and 48-55, Bauer et al. (1995) *Scand. J. Immunol. 42*: 317-323) of human papillomavirus type 16, the M2 protein of respiratory syncytial virus (residues 82-90 and 81-95, Hsu et al. (1995) *Immunology 85*: 347-350), the herpes simplex virus type 1 ribonucleotide reductase (see, Salvucci et al. (1995) *J. Gen. Virol. 69*: 1122-1131) and the rotavirus VP7 protein (see, Franco et al. (1993) *J. Gen. Virol. 74*: 2579-2586), *P. falciparum* antigens (causing malaria) and hepatitis B surface antigen (Gilbert et al. (1997) *Nature Biotech.* 15: 1280-1283).

Accordingly, sequences coding for the above-described proteins may be cloned into the present expression vector for delivery.

A number of coding sequences for small antigenic peptides can also be employed in the present invention. One skilled in the art can readily determine the length of the fragments required to produce immunogenic peptides. Alternatively, the skilled artisan can also use coding sequences for peptides that are known to elicit specific T cell responses, such as tumor-associated antigenic peptides (TRAs) as disclosed by U.S. Patent No. 5,462,871, U.S. Patent No. 5,558,995, U.S. Patent No. 5,554,724, U.S. Patent No. 5,585,461, U.S. Patent No. 5,591,430, U.S. Patent No. 5,554,506, U.S. Patent No. 5,487,974, U.S. Patent No. 5,530,096, U.S. Patent No. 5,519,117. Examples of TRAs are provided in Table 1. See also review by Van den Eynde and van der Bruggen (1997) and Shawler et al. (1997). Antigenic peptides of a pathogen origin can also be used, such as those disclosed by Gilbert et al. (1997).

As described herein above, sequences coding for a full-length naturally occurring protein, a part of a naturally occurring protein, combinations of parts of a naturally occurring protein, or combinations of different naturally occurring proteins or parts from different proteins, may all be employed in the present invention. For example, a sequence coding for multiple epitopes may be used, such as those described by Thomson et al. (1995). Preferably, the second DNA sequence of the present expression

vector codes for at least one epitope of a protein. An "epitope" refers to a peptide of at least 8 or 9 amino acids.

Those skilled in the art are familiar with the techniques to make DNA fragments coding for a part of a protein, or link a DNA sequence encoding a part of one protein in frame to a DNA sequence encoding a part of another protein and the like.

The vectors of the instant invention may include other sequence elements such as a 3' termination sequence (including a stop codon and a poly A sequence), or a gene conferring a drug resistance which allows the selection of *Yersinia* transformants having received the instant vector.

The expression vectors of the present invention may be transformed by a number of known methods into *Yersinia*. For the purpose of the present invention, the methods of transformation for introducing an expression vector include, but are not limited to, electroporation, calcium phosphate mediated transformation, conjugation, or combinations thereof. For example, a vector can be transformed into a first bacteria strain by a standard electroporation procedure. Subsequently, such a vector can be transferred from the first bacteria strain into *Yersinia* by conjugation, a process also called "mobilization". *Yersinia* transformant (i.e., *Yersinia* having taken up the vector) may be selected, e.g., with antibiotics. These techniques are well known in the art. See, for example, Sory et al. (1994).

One preferred embodiment of the present invention is directed to a *Yersinia* of the above-described mutant *Yersinia* strain transformed with an expression vector for delivery of a heterologous protein as herinabove described into a eukaryotic cell.

Accordingly, the present invention contemplates a method for delivering heterologous proteins as hereinabove described into eukaryotic cells.

The present invention contemplates a wide range of eukaryotic cells that may be targeted by the instant recombinant *Yersinia*.

By "target", is meant the extracellular adhesion of *Yersinia* to a eukaryotic cell.

In particular, the present invention contemplates antigen-presenting cells. "Antigen presenting cells" as referred herein express at least one class I or class II MHC determinant and may include those cells which are known as professional antigen-presenting cells such as macrophages, dendritic cells and B cells. Other professional antigen-presenting cells include monocytes, marginal zone Kupffer cells, microglia, Langerhans' cells, interdigitating dendritic cells, follicular dendritic cells, and T cells. Facultative antigen-presenting cells can also be used according to the present invention. Examples of facultative antigen-presenting cells include astrocytes, follicular cells, endothelium and fibroblasts. As used herein,

"antigen-presenting cells" encompass both professional and facultative types of antigen-presenting cells.

The antigen presenting cells can be isolated from tissue or blood (containing peripheral blood mononuclear cells) samples obtained from a mammal such as a human or rodent. Cell lines established from such samples may also be used. Procedures for establishing cell lines are well known in the art. Certain cell lines may be obtained directly from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852-1776. Both normal and malignant cells may be employed.

In accordance with a preferred embodiment of the present invention, the MHC determinants expressed by the antigen presenting cell are compatible with those expressed by the mammal involved, and at least one of these MHC determinants is capable of presenting one or more antigenic epitopes derived from the protein being delivered.

One skilled in the art is also familiar with the methods for determining whether the MHC molecules expressed by the antigen presenting cell are compatible with those of the mammal subject involved, such as well known HLA-typing procedures. See general teachings by Coligan et al. (1994) *Current Protocols in Immunology* John Wiley & Sons Inc: New York, New York.

Those skilled in the art are able, through the extensive teachings in the art, to determine the MHC molecule for presentation of a particular antigen. For example, U.S. Patent No. 5,405,940 teaches the

determination of HLA-A1 as the presenting molecule for a peptide of MAGE-1, EADPTGHSY; U.S. Patent No. 5,558,995 teaches the determination of HLA-Cw1601 for presenting another peptide of MAGE-1, SAYGEPRKL; U.S. Patent No. 5,530,096 teaches the determination of HLA-A2 as the presenting molecule for a peptide of Tyrosinase, MLLAVLYCL. In the event the eukaryotic cells being targeted do not express a desired HLA or MHC molecule, the gene encoding such molecule may be introduced into the eukaryotic cells by well known transformation or transfection procedures.

Further in accordance with the present invention, the delivery of a protein can be achieved by contacting a eukaryotic cell with a recombinant *Yersinia* under appropriate conditions. Various references and techniques are conventionally available for those skilled in the art regarding the conditions for inducing the expression and translocation of virulon genes, including the desired temperature, Ca++ concentration, manners in which *Yersinia* and target cells are mixed, and the like. See, for example, Cornelis, *Cross talk between Yersinia and eukaryotic cells*, Society for General Microbiology Symposium, 55; MoCRAE, SAUNDERS, SMYTH, STOW (eds), *Molecular aspects of host-pathoge interactions*, Cambridge University Press, 1997. The conditions may vary depending on the type of eukaryotic cells to be targeted, e.g., the conditions for targeting human epithelial carcinoma Hela cells (Sory et al. (1994)); the conditions for targeting mouse thymoma or melanoma cells (Starnbach et al. (1994) *J. Immunol. 153*: 1603);.the conditions

for targeting mouse macrophages (Boland et al. (1996)). Such variations can be addressed by those skilled in the art using conventional techniques.

Those skilled in the art can also use a number of assays to determine whether the delivery of a fusion protein is successful. For example, the fusion protein may be labeled with an isotope or an immunofluoresceine, or detected by a immunofluoresceine conjugated antibody, as disclosed by Rosqvist et al. (1994) *EMBO J. 13*: 964. The determination can also be based on the enzymatic activity of the protein being delivered, e.g., the assay described by Sory et al. (1994). The determination can also be based on the antigenicity of the protein being delivered. For example, the delivery of a MAGE-1 protein into EBV-transformed human B cells can be detected by the recognition of such targeted B cells by CTL cells specific for MAGE-1 epitopes. Such CTL recognition, in turn, may be detected by a number of assays including assaying the secretion of IFN-γ from the activated CTLs or Cr^{51} release from lysed target cells. Methods such as Western-blot analysis using antibodies specific against the protein being delivered, PCR in situ hybridization, or ELISPOT (Mabtech AB, Sweden) may also be employed for such determination. See, e.g., W. Herr et al. (1997) *J. Immunol. Methods 203*: 141-152 and W. Herr et al. (1996) *J. Immunol. Methods 191*: 131-142.

In a further aspect of the present invention, recombinant *Yersinia* capable of delivering

proteins to antigen-presenting cells are employed for inducing an immune response. Accordingly, the present invention contemplates immunogenic compositions and methods for inducing specific immune responses using the instant recombinant *Yersinia* as described hereinabove.

The immune responses contemplated by the present invention include cellular immune responses (mediated primarily by T cells) and humoral immune responses (mediated primarily by antibodies). Janeway and Travers teach in general these immune response. (Janeway and Travers (1996) *Immunology, The Immune System in Health and Disease* 2nd ed. Garland Publishing, Inc.: New York, New York, and London, England.) (See also, review by O. Tureci et al. (1997) *Molecular Medicine Today 3(8)*: 342-349.

According to this aspect of the present invention, the immune responses induced with recombinant *Yersinia* can be utilized in a number of regimes for diagnostic or therapeutic use. For example, recombinant *Yersinia* can be employed in an *in vitro* procedure for monitoring the efficacy of a vaccination therapy in a mammal such as a human or rodent. In this regime, certain antigen presenting cells (e.g., dendritic cells) are taken from a subject being vaccinated with immunogenic compositions, e.g., a particular antigen. Such antigen presenting cells are then contacted with recombinant *Yersinia* capable of delivering the antigen which is used for vaccination. Subsequently, peripheral blood lymphocytes taken from the same subject (i.e.,

autologous PBLs) are added, preferably in combination with cytokines such as IL-2, to the mixture of antigen presenting cells and *Yersinia*. The efficacy of the vaccination can be assessed after priming and then after successive boosts by the presence of CTLs or antibodies that are specific for the relevant antigen. The presence of specific CTLs can be detected using standard assays such as an assay for Cr^{51} release or for the secretion of IFN-gamma. The presence of specific antibodies can be detected by assays such as ELISA using the antigens which are immobolized on a culture plate, or a standard proliferation assay for T-helper cells.

Recombinant *Yersinia* can also be employed in an *ex vivo* regime for inducing CTLs specific for a protein. The procedure to develop such specific CTLs *in vitro* is known in the art, e.g., as disclosed by the United States Patent No. 5,342,774. Briefly, a blood sample containing T cell precursors is taken from a mammal. PBLs are purified from such blood sample and are incubated with stimulator cells expressing an antigenic epitope in the context of an MHC molecule. CTLs specific for such epitope produced can be detected by assays such as an assay for Cr^{51} release or secretion of IFN-gamma.

According to the present invention, a mixture of a recombinant *Yersinia* and an antigen presenting cell can be used as the "stimulator cell" in such an *in vitro* procedure for producing CTLs specific for the protein being delivered. The MHC determinants expressed by the antigen presenting cell

used are compatible with those expressed by the mammal from which PBLs are isolated, and at least one of these MHC molecules is capable of presenting, to T cells, one or more epitopes derived from the protein being delivered. CTL cells generated as such can be administered, in a therapy regimen of adoptive transfer, to a mammal a pathological condition characterized by an abnormal expression of the protein used in the delivery system. See teachings by Greenberg (1986) *J. Immunol. 136 (5)*: 1917; Riddel et al. (1992) *Science 257*: 238; Lynch et al. (1991) *Eur. J. Immunol. 21*: 1403; and Kast et al. (1989) *Cell 59*: 603 for adoptive transfer. CTLs, by lysing the cells abnormally expressing such antigens, can alleviate or treat the pathological condition at issue such as a tumor, an infection with a parasite or a virus.

Accordingly, the present invention contemplates methods and compositions for treating pathological conditions. The pathological conditions contemplated by the present invention include tumors and infections by pathogens such as bacteria, parasites, fungus or virus.

By "treating", is meant alleviating or inhibiting a pathological condition, e.g., inhibiting tumor growth or metastasis, reducing the size of tumor, or diminishing symptoms of a pathogen infection.

The recombinant *Yersinia* of the present invention can also be employed *in vivo*, i.e., introducing recombinant *Yersinia* into a mammal, such as a human or rodent subject.

For in vivo use of recombinant *Yersinia*, the safety can be tested in animals beforehand. In this case, the recombinant *Yersinia* may be administered to the animal orally or directly into the stomach. The animals may be sacrificed a few days (1-3 days) after the administration of the recombinant *Yersinia*. The intestines are washed and the Peyer patches or the faeces can be examined for viable *Yersinia*. See, e.g., Sory et al. (1992) *Infect. Immun. 60*: 3830-3836. The recombinant *Yersinia* may also be administered to the animal by intraperitoneal injection. Organs of sacrificed animals such as spleen and liver can be examined for the presence of intracellular *Yersinia*, an indication of insufficient safety. Intracellular *Yersinia* may be detected by e.g., cultivating cell extracts on solid medium. See teachings by Sory et al. (1988) *Microb. Pathogen 4*: 431-442.

A safe recombinant *Yersinia* may be employed in an immunogenic composition to induce an immune response for treating various pathological conditions in mammals. The pathological conditions contemplated by the present invention include tumors and pathogen infections, as disclosed herein.

The immunogenic compositions can include, in addition to a recombinant *Yersinia*, other substances such as cytokines, adjuvants and pharmaceutically acceptable carriers. Cytokines can also be included in such immunogenic composiitons using additional recombinant *Yersinia* of the present invention capable of delivering a cytokine, for example.

These immunogenic compositions may be administered to the subject in any convenient manner, such as orally, intraperitoneally, intravenously or subcutaneously. Specific immune responses induced by such compositions can lead to the CTL-mediated or antibody-mediated killing of the pathogens or cells with abnormal expression of a relevant antigen, thus alleviating the relevant pathological condition.

The present invention is further illustrated by the following examples.

All the publications mentioned in the present disclosure are incorporated herein by reference. The terms and expressions which have been employed in the present disclosure are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

Example 1

Bacterial Strains, Plasmids and Growth Conditions

The work was carried out with *Y.enterocolitica* E40(pYV40) (see, M.P. Sory et al. (1995) "Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the cyaA gene fusion approach" *Proc. Nat'l Acad. Sci. USA 92*: 11998-12002), its isogeneic ampicillin sensitive derivative MRS40(pYV40) (see, M.R. Sarker et al., and their various non-polar mutants. Plasmids are listed in Table 1. Bacteria were grown in Brain Heart Infusion (BHI) (Difco, Destroit, Michigan). After overnight preculture, bacteria were diluted 1/20 in fresh BHI, allowed to grow for 30 minutes at room temperature, and synthesis of the Yop virulon was induced by incubation for 150 minutes at 37°C before infection.

Table 1: Exemplary Antigens

Gene	MHC	Peptide	Position	SEQ ID NO:
MAGE-1	HLA-A1	EADPTGHSY	161-169	1
	HLA-Cw16	SAYGEPRKL	230-238	2
MAGE-3	HLA-A1	EVDPIGHLY	168-176	3
	HLA-A2	FLWGPRALV	271-279	4
	HLA-B44	MEVDPIGHLY	167-176	5
BAGE	HLA-Cw16	AARAVFLAL	2-10	6
GAGE-1,2	HLA-Cw16	YRPRPRRY	9-16	7
RAGE	HLA-B7	SPSSNRIRNT	11-20	8
GnT-V	HLA-A2	VLPDVFIRC(V)	2-10/11	9
MUM-1	HLA-B44	EEKLIVVLF	exon 2/ intron	10
		EEKLSVVLF (wild type)		11
CDK4	HLA-A2	ACDPHSGHFV	23-32	12
		ARDPHSGHFV (wild type)		13
β-catenin	HLA-A24	SYLDSGIHF	29-37	14
		SYLDSGIHS (wild type)		15
Tyrosinase	HLA-A2	MLLAVLYCL	1-9	16
	HLA-A2	YMNGTMSQV	369-377	17
	HLA-A2	YMDGTMSQV	369-377	18
	HLA-A24	AFLPWHRLF	206-214	19

	HLA-B44	SEIWRDIDF	192-200	20
	HLA-B44	YEIWRDIDF	192-200	21
	HLA-DR4	QNILLSNAPLGPQ FP	56-70	22
	HLA-DR4	DYSYLQDSDPDSF QD	448-462	23
Melan-A^{MART-1}	HLA-A2	(E)AAGIGILTV	26/27-35	24
	HLA-A2	ILTVILGVL	32-40	25
$gp100^{Pmel117}$	HLA-A2	KTWGQYWQV	154-162	26
	HLA-A2	ITDQVPFSV	209-217	27
	HLA-A2	YLEPGPVTA	280-288	28
	HLA-A2	LLDGTATLRL	457-466	29
	HLA-A2	VLYRYGSFSV	476-485	30
DAGE	HLA-A24	LYVDSLFFL	301-309	31
MAGE-6	HLA-Cw16	KISGGPRISYPL	292-303	32

Example 2

Construction of the Polymutant Strains

To construct the *yopHOPEM* polymutant strain, the *yopE*, *yopH*, *yopO*, *yopM* and *yopP* genes were successively knocked out by allelic exchange in the MRS40 strain using the suicide vectors pMRS101 and pKNG101. See, K. Kaniga et al. (1991) "A wide-host range suicide vector for improving reverse genetics in gram-negative bacteria: inactivation of the blaA gene of *Yersinia enterocolitica*" *Gene 109*: 137-141 and M.R. Sarker et al. (1997) "An improved version of suicide vector pKNG101 for gene replacement in Gram-negative bacteria" *Mol. Microbiol. 23*: 409-411. The various deletions are described in Table 2 in the "suicide vectors and mutators" section. The *YopE* gene was first mutated using the mutator pPW52 (see, P. Wattiau et al. (1993) "SycE, a chaperone-like protein of *Yersinia enterocolitica* involved in the secretion of YopE" *Mol. Microbiol. 8*: 123-131), giving strain MRS40(pAB4052). Mutation of the *YopH* gene in this strain with the mutator pAB31 (see, S.D. Mills et al. (1997) "*Yersinia enterocolitica* induces apoptosis in macrophages by a process requiring functional type III secretion and translocation mechanisms and involving YopP, presumably acting as an effector protein" *Proc. Natl. Acad. Sci. USA 94*: 12638-12643) gave the double *yopEH* mutant MRS40(pAB404). The triple *yopEHO* mutant MRS40(pAB405) was then obtained by allelic exchange with the mutator pAB34 (see, S.D. Mills et al., 1997). The *YopP* gene was then mutated with mutator pMSK7 (see

S.D. Mills et al. (1997)), leading to the *yopEHOP* mutant MRS40(pMSK46). The *yopHOPEM* strain MRS40(pABL403) was finally obtained by allelic exchange with the *yopM* mutator pAB38 (see, S.D. Mills et al., 1997).

Table 2: Plasmids

Plasmids	Relevant Characteristics	References
pYV		
pABL403	pYV40 $yopE_{21}$, $yopH\Delta^{1-352}$ $yopO\Delta^{65-558}$, $yopP_{23}$, $yopM_{23}$	see Example 2 of the present specification
	Suicide Vectors and mutators	
pKNG101	^{ori}R6K ^{sac}BR+ ^{on}TRK2 ^{str}AB+	K. Kaniga et al. (1991) *Gene 109*: 137-141.
pMRS101	^{ori}R6K ^{sac}BR+ ^{on}TRK2 ^{str}AB+ ^{ori}ColE1 *bla* +	M.R. Sarker and G.R. Cornelis (1997) *Mol. Microbiol. 23*: 409-411.
pAB31	pMRS101 $yopH\Delta_{1-352}$+	S.D. Mills et al. (1997) *Proc. Natl. Acad. Sci. USA 94*: 12638-12643.
pAB34	pMRS101 $yopO\Delta_{65-558}$+	S.D. Mills et al. (1997)
pAB38	pMRS101 $yopM_{23}$+	S.D. Mills et al. (1997)
pMSK7	pMRS101 $yopP_{23}$+	S.D. Mills et al. (1997)
pPW52	pKNG101 $yopE_{21}$+	P. Waattiau and G.R. Cornelis (1993) *Mol. Microbiol. 8*: 123-131.

Example 3

Construction of a Plasmid Encoding $YopE_{130}$-MAGE-1 and Introduction of this Plasmid into *Yersinia*

The sequence encoding protein MAGE-1 was inserted in frame with a sequence encoding a truncated YopE, $YopE_{130}$, containing the first 130 amino acids of YopE. Such a plasmid is graphically depicted in Figure 1.

The open reading frame of MAGE-1 was amplified by PCR using a MAGE-1 cDNA cloned in pcDNAI/Amp (Invitrogen, Carlsbad, California) as template. The upstream primer, AAACTGCAGATGTCTCTTGAGCAGAGGAGTC, consisted of the first nucleotides of the open reading frame of MAGE-1 preceded by a PstI site. The downstream primer, AAACTGCAGTCAGACTCCCTCTTCCTCCTC, consisted of nucleotides complementary to the last nucleotides of the open reading frame of MAGE-1 followed by a PstI site. The PCR product was digested with PstI and inserted in frame with the truncated *YopE* at the PstI site of vector pMS111 (see, Sory et al. (1994) *Molecular Microbiology 14*: 583-594). pMS111-MAGE-1 was electroporated in bacteria strain DH5αF'IQ. DNA was extracted from some clones and the DNA of a positive recombinant clone was electroporated in bacteria strain SM10. After mobilization of pMS111 from SM10 in *Yersinia* MRS40 (pABL403), recombinant clones were then selected on agar-containing medium, supplemented with nalidixic acid, sodium-arsenite and chloramphenicol. MRS40 is an isogeneic derivative of

E40 sensitive to ampicillin (see, Sory et al. (1995) *Proc. Natl. Acad. Sci. USA 92*: 11998-12002).

Example 4

Targeting EBV-Transformed B Cells

One colony of *Yersinia* MRS40 (pABL403) containing pMS111-MAGE-1 was then grown overnight at 28°C in LB medium supplemented with nalidixic acid, sodium m-arsenite and chloramphenicol. The overnight culture was diluted in fresh medium in order to obtain an OD (optical desity) of 0.2. The fresh culture was amplified at 28°C for approximately 2 hours. The bacteria were washed in 0.9% NaCl and resuspended at 10^8 bacteria per ml in 0.9% NaCl. 50,000 EBV-transformed HLA-A1$^+$ B cells (KASOII-EBV) were placed in microwells (96 wells round-bottomed) and pelleted by centrifugation. The supernatant was discarded and various dilutions of bacteria were added in 100 ul of complete RPMI 1640 (culture media was supplemented with 10% FCS and with L-arginine (116 mg/ml), L-asparagine (36 mg/ml), L-glutamine (216 mg/ml). Two hours after infection, gentamicin (30 μg/ml) was added for the next two hours, and the cells were finally washed three times.

As a negative control, the same cells were also infected with *Yersinia* MRS40 (pABL403) containing pMS621, a plasmid which encodes only the truncated YopE, i.e., $YopE_{130}$.

Example 5

Recognition of Targeted B Cells by MZ2-CTL 82/30

MZ2-CTL 82/30 are specific for the MAGE-1 peptide EADPTGHSY which is presented by HLA-A1 (U.S. Patent No. 5,342,774). 5000 MZ2-CTL 82/30 cells were added in each microwell containing the *Yersinia* in a final volume of 100 μl of Iscove's complete medium (culture medium was supplemented with 10% human serum, L-arginine (116 mg/ml), L-asparagine (36 mg/ml), L-glutamine (216 mg/ml), streptomycine (0.1 mg/ml), penicillin (200 U/ml), IL-2 (25 U/ml) and gentamicin (15 μg/ml). After overnight incubation, the presence of IFN-gamma (that is produced by CTL upon activation) in the supernatant of the co-culture was tested in a standard ELISA assay (Biosource, Fleurus, Belgium). Figure 2A graphically depicts such a procedure.

As indicated in Figure 2B, the HLA-A1$^+$ B cells infected with *Yersinia* encoding YopE$_{130}$-MAGE-1 were recognized by the CTL 82/30, while the same cells infected with the control plasmid YopE$_{130}$ were not. The optimal concentration of bacteria is around 1,000,000 per microwell.

WHAT IS CLAIMED IS:

1. A mutant *Yersinia* strain comprising at least one mutation in at least one *Yersinia* effector-encoding gene, whereby said mutant *Yersinia* strain is deficient in the production of at least one functional effector protein.

2. The mutant *Yersinia* strain according to claim 1, wherein said effector encoding gene is selected from the group consisting of *YopE*, *YopH*, *YopO*, *YopM* and *YopP* of *Y. enterocolitica*; and *YopE*, *YopH*, *YpkA*, *YopM* and *YopJ* of *Y. pseudotuberculosis*.

3. The mutant *Yersinia* strain according to claim 1, wherein said mutation is a mutation of the promoter sequence of said effector gene.

4. The mutant *Yersinia* strain according to claim 1, wherein said mutation is a mutation of the coding sequence of said effector gene.

5. A quintuple mutant *Yersinia* strain having the designation of *Y. enterocolitica yopEHOMP* or *Y. pseudotuberculosis yopEHAOJ*.

6. The quintuple mutant *Yersinia* strain according to claim 5, having the designation of *Yersinia enterocolitica* MRS40(pABL403).

7. An expression vector for delivering a heterologous protein into a eukaryotic cell, which comprises in the 5' to 3' direction:

a promoter from a *Yersinia* virulon gene;

a first DNA sequence encoding a delivery signal from a *Yersinia* effector protein, operably linked to said promoter; and

a second DNA sequence coding for said heterologous protein, fused in frame to the 3' end of said first DNA sequence.

8. The expression vector of claim 7, wherein said *Yersinia* virulon gene is a *Yersinia* effector-encoding gene.

9. The expression vector of claim 8, wherein said effector-encoding gene is selected from the group consisting of *YopE*, *YopH*, *YopO*, *YopM* and *YopP of Y. enterocolitica*; and *YopE*, *YopH*, *YpkA*, *YopM* and *YopJ* of *Y. pseudotuberculosis*.

10. The expression vector of claim 9, wherein said effector-encoding gene is *Y. enterocolitica* of *YopE*.

11. The expression vector of claim 7, wherein said effector protein is selected from the group consisting of YopE, YopH, YopO, YopM and YopP of *Yersinia enterocolitica*; and YopE, YopH, YpkA, YopM and YopJ of *Y. pseudotuberculosis*.

12. The expression vector of claim 11, wherein said effector protein is one of *Yersinia enterocolitica* YopE or *Y. pseudotuberculosis* YopE.

13. The expression vector of claim 7, wherein said delivery signal is *Y. enterocolitica* $YopE_{130}$.

14. The expression vector of claim 7, wherein said heterologous protein comprises at least one epitope of a naturally occurring protein.

15. The expression vector of claim 14, wherein said naturally occurring protein is a tumor associated protein or a pathogen antigen.

16. The expression vector of claim 15, wherein said tumor associated protein is selected from the group consisting of members of the MAGE family, the BAGE family, the DAGE/Prame family, the GAGE family, the RAGE family, the SMAGE family, NAG, Tyrosinase, Melan-A/MART-1, gp100, MUC-1, TAG-72, CA125, p21ras, p53, HPV16 E7,HOM-MEL-40, HOM-MEL-55, NY-COL-2, HOM-HD-397, HOM-RCC-1.14, HOM-HD-21, HOM-NSCLC-11, HOM-MEL-2.4, and HOM-TES-11.

17. The expression vector of claim 16, wherein said tumor-associated protein is MAGE-1.

18. A *Yersinia* of a *Yersinia* mutant strain of any one of claims 1-6 for delivery of a

heterologous protein into a eukaryotic cell, wherein said *Yersinia* is transformed with an expression vector which comprises in the 5' to 3' direction:

a promoter from a *Yersinia* virulon gene;

a first DNA sequence encoding a delivery signal from a *Yersinia* effector protein, operably linked to said promoter; and

a second DNA sequence coding for said heterologous protein, fused in frame to the 3' end of said first DNA sequence.

19. The *Yersinia* according to claim 18, wherein said *Yersinia* virulon gene is a *Yersinia* effector-encoding gene.

20. The *Yersinia* according to claim 19, wherein said effector-encoding gene is selected from the group consisting of *YopE*, *YopH*, *YopO*, *YopM* and *YopP of Y. enterocolitica*; and *YopE*, *YopH*, *YpkA*, *YopM* and *YopJ* of *Y. pseudotuberculosis*.

21. The *Yersinia* according to claim 20, wherein said effector-encoding gene is *Y. enterocolitica YopE*.

22. The *Yersinia* according to claim 18, wherein said effector protein selected from the group consisting of YopE, YopH, YopO, YopM and YopP of *Yersinia enterocolitica*; and YopE, YopH, YpkA, YopM and YopJ of *Y. pseudotuberculosis*.

23. The *Yersinia* according to claim 22, wherein said effector protein is one of *Yersinia enterocolitica*'s YopE or *Y. pseudotuberculosis*'s YopE.

24. The *Yersinia* according to claim 18, wherein said delivery signal is *Y. enterocolitica* $YopE_{130}$.

25. The *Yersinia* according to claim 18, wherein said heterologous protein comprises at least one epitope of a naturally occurring protein.

26. The *Yersinia* according to claim 25, wherein said naturally occurring protein is a tumor-associated protein or a pathogen antigen.

27. The *Yersinia* according to claim 26, wherein said tumor-associated protein is selected from the group consisting of members of the MAGE family, the BAGE family, the DAGE/Prame family, the GAGE family, the RAGE family, the SMAGE family, NAG, Tyrosinase, Melan-A/MART-1, gp100, MUC-1, TAG-72, CA125, p21ras, p53, HPV16 E7,HOM-MEL-40, HOM-MEL-55, NY-COL-2, HOM-HD-397, HOM-RCC-1.14, HOM-HD-21, HOM-NSCLC-11, HOM-MEL-2.4, and HOM-TES-11.

28. The *Yersinia* according to claim 27, wherein said tumor-associated protein is MAGE-1.

29. A method for delivering a heterologous protein into a eukaryotic cell, comprising contacting

said eukaryotic cell with a *Yersinia* of a mutant strain of claim 1 transformed with an expression vector characterized by in 5' to 3' direction:

a promoter from a *Yersinia* virulon gene;

a first DNA sequence encoding a delivery signal from a *Yersinia* effector protein, operably linked to said promoter; and

a second DNA sequence coding for said heterologous protein, fused in frame to the 3' end of said first DNA sequence.

30. The method of claim 29, wherein said eukaryotic cell is an antigen presenting cell.

31. The method of claim 30, wherein said antigen presenting cell is selected from the group consisting of a B cell, a macrophage, a dendritic cell, a monocyte, a follicular cell, and a fibroblast.

32. The method of claim 30, wherein said antigen presenting cell expresses an MHC molecule capable of presenting one or more epitopes derived from said heterologous protein.

33. The method of claim 29, wherein said effector protein is selected from the group consisting of YopE, YopH, YopO, YopM and YopP of *Yersinia enterocolitica*; and YopE, YopH, YpkA, YopM and YopJ of *Y. pseudotuberculosis*.

34. The method of claim 33, wherein said effector protein is one of *Yersinia enterocolitica* yopE or *Y. pseudotuberculosis* YopE.

35. The method of claim 29, wherein said effector protein is $YopE_{130}$ of *Yersinia enterocolitica*.

36. The method of claim 29, wherein said heterologous protein comprises at least one epitope of a naturally occurring protein.

37. A *Yersinia* according to claim 36, wherein said naturally occurring protein is a tumor associated protein or a pathogen antigen.

38. The expression vector of claim 37, wherein said tumor associated protein is selected from the group consisting of members of the MAGE family, the BAGE family, the DAGE/Prame family, the GAGE family, the RAGE family, the SMAGE family, NAG, Tyrosinase, Melan-A/MART-1, gp100, MUC-1, TAG-72, CA125, p21ras, p53, HPV16 E7,HOM-MEL-40, HOM-MEL-55, NY-COL-2, HOM-HD-397, HOM-RCC-1.14, HOM-HD-21, HOM-NSCLC-11, HOM-MEL-2.4, and HOM-TES-11.

39. The method of claim 38, wherein said tumor-associated protein is MAGE-1.

40. The method of claim 29, wherein said *Yersinia* virulon gene is a *Yersinia* effector-encoding gene.

41. The method of claim 40, wherein said effector-encoding gene is selected from the group consisting of *YopE*, *YopH*, *YopO*, *YopM* and *YopP of Y. enterocolitica*; and *YopE*, *YopH*, *YpkA*, *YopM* and *YopJ* of *Y. pseudotuberculosis*.

42. The method of claim 41, wherein said effector-encoding gene is *YopE* of *Y. enterocolitica*.

43. A method for inducing an immune response specific for a heterologous protein, comprising.the steps of:

(a) selecting an antigen presenting cell expressing an MHC molecule capable of presenting at least one epitope of said heterologous protein;

(b) forming a cell mixture by contacting said antigen presenting cell with a *Yersina* of a mutant *Yersinia* strain of claim 1 transformed with an expression vector thereby delivering said heterologous protein into said antigen presenting cell, wherein said expression vector is characterized by in 5' to 3' direction:

a promoter from a *Yersinia* virulon gene;

a first DNA sequence encoding a delivery signal from a *Yersinia* effector protein, operably linked to said promoter;

a second DNA sequence coding for said heterologous protein, fused in frame to the 3' end of said first DNA sequence; and

(c) contacting a sample containing peripheral blood lymphocytes taken from a subject,

with the cell mixture formed in step (b) thereby inducing an immune response specific for said heterologous protein.

44. The method of claim 43, wherein said epitope of is from a tumor associated protein.

45. The method of Claim 44, wherein said tumor associated protein is selected from the group consisting of members of the MAGE family, the BAGE family, the DAGE/Prame family, the GAGE family, the RAGE family, the SMAGE family, NAG, Tyrosinase, Melan-A/MART-1, gp100, MUC-1, TAG-72, CA125, p21ras, p53, HPV16 E7,HOM-MEL-40, HOM-MEL-55, NY-COL-2, HOM-HD-397, HOM-RCC-1.14, HOM-HD-21, HOM-NSCLC-11, HOM-MEL-2.4, and HOM-TES-11.

46. The method of claim 45, wherein said tumor associated protein is MAGE-1.

47. The method of claim 46, wherein said epitope is from MAGE-1 and said MHC molecule is HLA-A1.

48. An immunogenic composition, comprising a recombinant *Yersinia* according to claim 18.

49. A method of inducing a CTL response specific for a heterologous protein in a subject in need of such response, comprising.the steps of:

(a) obtaining from said subject an antigen presenting cell expressing an MHC molecule;

(b) forming a cell mixture by contacting said antigen presenting cell with a *Yersina* of a mutant *Yersinia* strain of claim 1 transformed with an expression vector, wherein said expression vector is characterized by in 5' to 3' direction:

a promoter from a *Yersinia* virulon gene;

a first DNA sequence encoding a delivery signal from a yersinia effector protein, operably linked to said promoter; and

a second DNA sequence fused in frame to the 3' end of said first DNA sequence, wherein said second DNA sequence codes for at least one epitope of said heterologous protein which is presented by said MHC molecule of said antigen presenting cell;

(c) contacting a sample containing peripheral blood lymphocytes taken from said subject, with the cell mixture formed in step (b), thereby producing CTLs specific for said heterologous protein; and

(d) administering CTLs produced in step (c) to said subject thereby inducing a CTL response specific for said heterologous protein in said subject.

50. A method for determining the efficacy of a vacciantion regimen in a subject, wherein said subject is vaccinated with an antigen, comprising the steps of:

(a) obtaining from said subject an antigen presenting cell expressing an MHC molecule;

(b) forming a cell mixture by contacting said antigen presenting cell with a *Yersina* of a mutant *Yersinia* strain of claim 1 transformed with an expression vector, wherein said expression vector is characterized by in 5' to 3' direction:

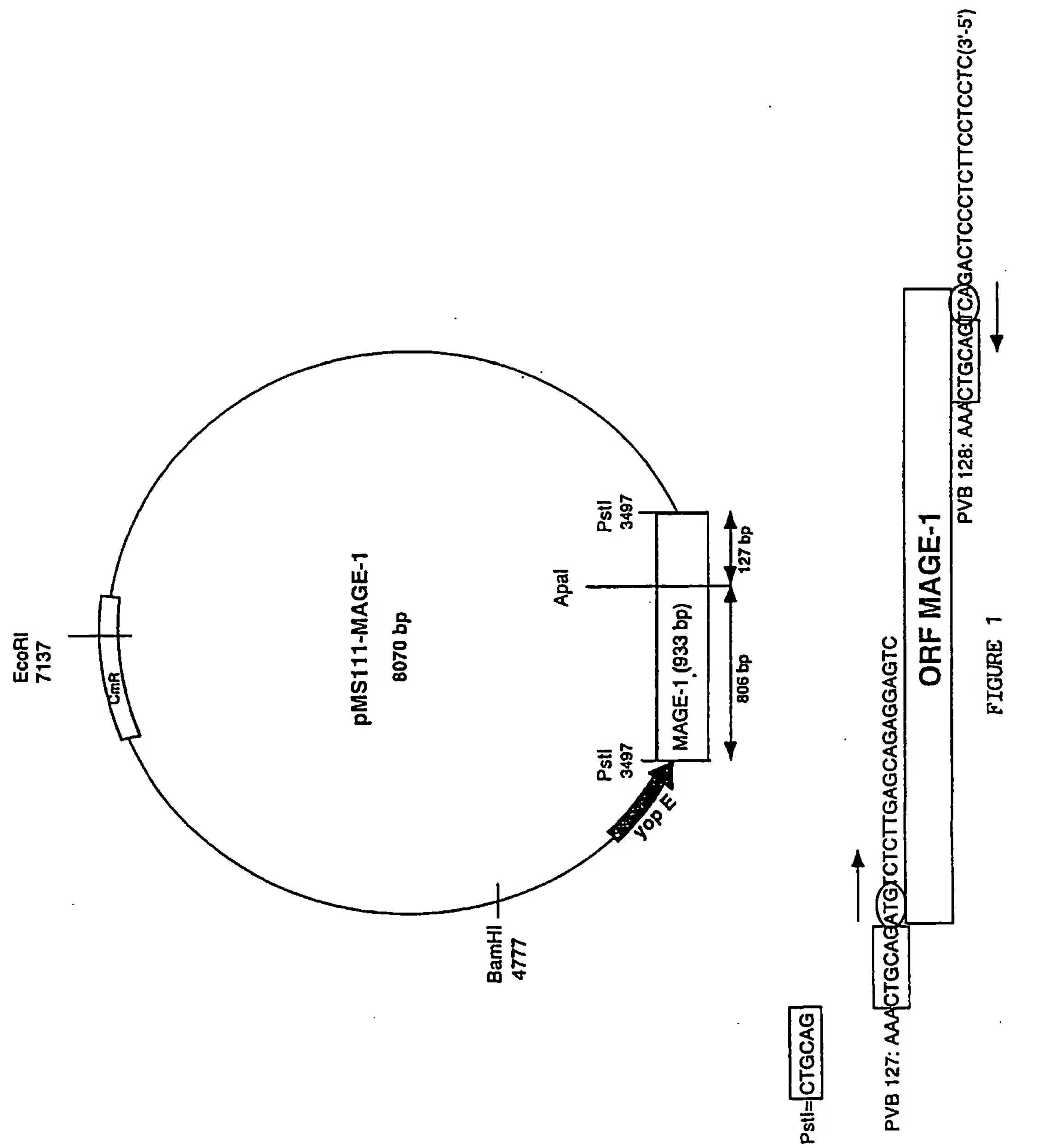
a promoter from a *Yersinia* virulon gene;

a first DNA sequence encoding a delivery signal from a yersinia effector protein, operably linked to said promoter; and

a second DNA sequence fused in frame to the 3' end of said first DNA sequence, wherein said second DNA sequence codes for at least one epitope of said antigen which is presented by said MHC molecule of said antigen presenting cell;

(c) contacting a sample containing peripheral blood lymphocytes taken from said subject, with the cell mixture formed in step (b), and assaying the presence of an immune response specific for said antigen thereby determining the efficacy of said vaccination regimen.

51. A method for treating a pathological disorder in a subject, comprising administering the yersinia of claim 18 to said subject, wherein the heterologous protein elicits an immune response specific to said pathological disorder.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CmR
pMS111-MAGE-1
8070 bp
BamHI
4777
yop E
PstI
3497
ApaI
PstI
3497
MAGE-1 (933 bp)
127 bp
806 bp
PstI=CTGCAG
PVB 127: AACTGCAGATGTCTCTTGAGCAGAGGAGTC
ORF MAGE-1
PVB 128: AACTGCAGTCAGACTCCCTCTTCCTCCTC(3'-5')

FIGURE 1

Anti-MAGE-1.A1 CTL recognize HLA-A1 cells incubated with Yersinia which produces a YopE130.MAGE-1 fusion protein

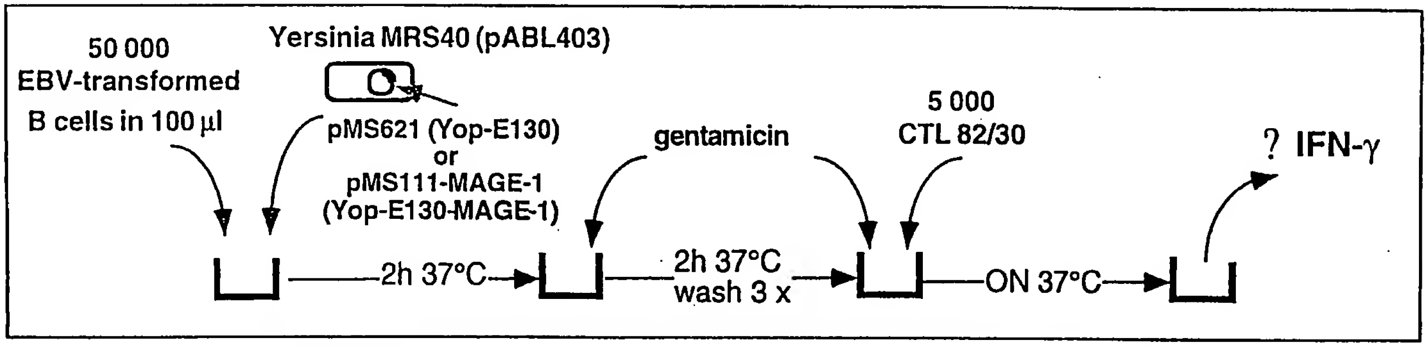

FIGURE 2A

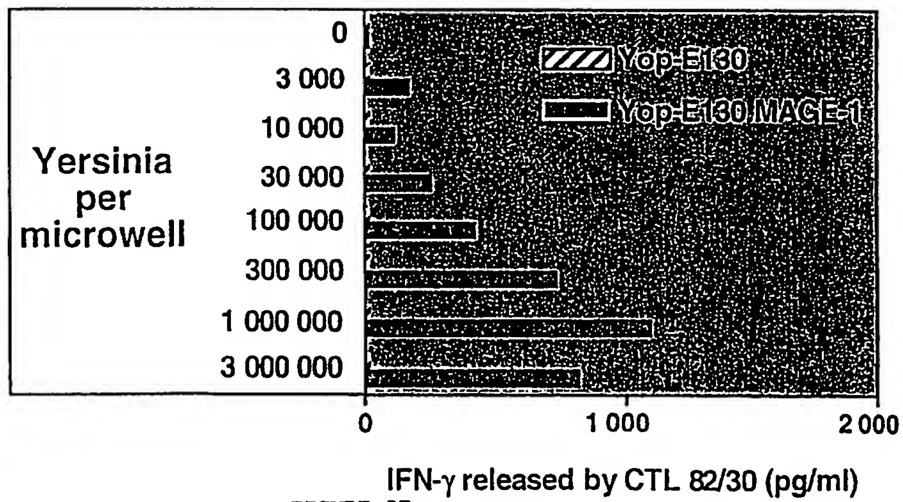

FIGURE 2B

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181  atgtatctaa tacttttttg caagaaccat tacgtcattc ttctgattta actgagatgc
241  cagttgaggc agaaaatgtt aaatctaagg ctgaatatta taatgcatgg tcggaatggg
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361  attgcctgga ccgacaagcc catgagctag aactaaataa tctggggctg agttctttgc
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FIGURE 3

```
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FIGURE 4

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301  cttaatttaa ttaaggaggg aagcatgaac ttatcattaa gcgatcttca tcgtcaggta
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FIGURE 5

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FIGURE 6

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FIGURE 7

SEQUENCE LISTING

<110> van der Bruggen, Pierre
Cornelis, Guy R.

<120> DELIVERY OF PROTEINS INTO EUKARYOTIC CELLS
WITH RECOMBINANT *YERSINIA*

<130> 11154

<140> US 09/036,582

<141> 1998-03-06

<160> 39

<170> PatentIn Ver. 2.0

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<210> 26

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<210> 28

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<213> Human gp100^{Pmel117} peptide

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<210> 30

<211> 10

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<213> Human gp100[Pmel117] peptide

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<213> Human MAGE-6 peptide

<400> 32

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<211> 1330

<212> DNA

<213> *Yersinia enterocolitica*

<400> 33

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CGGAATTACC TCCGCATTTA GAGAGTTTAG TGGCGTCATG TAATTCTCTT ACAGAATTAC  480
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<212> DNA

<213> *Yersinia enterocolitica*

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<211> 1990

<212> DNA

<213> *Yersinia enterocolitica*

<400> 35

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<212> DNA

<213> *Yersinia enterocolitica*

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